# Oxygen flux reduces Cux1 positive neurons and cortical growth in a gestational rodent model of growth restriction

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# Title: Oxygen flux reduces Cux1 positive neurons and cortical growth in a gestational rodent model of growth restriction

**Running title: Cortical effects of oxygen and growth**

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## Abstract

**Background:** The mammalian cerebral cortex forms in an inside-out manner, establishing deep cortical layers before superficial layers and is regulated by transcription factors which influence cell differentiation. Preterm birth interrupts the trajectory of normal neurodevelopment and adverse perinatal exposures have been implicated in cortical injury. We hypothesise that growth restriction (GR) and fluctuating hyperoxia ($\Delta O_2$) impair cortical laminar development.

**Methods:** Sprague-Dawley rats received 18% (non-restricted, NR) or 9% (growth restricted, GR) protein diet from E15-P7. Litters were reared in air or fluctuating hyperoxia (circa 10kPa) from P0-P7. Cortical laminae were stained and measured. Neuronal subtypes were quantified using immunofluorescence for subtype-specific transcription factors (Satb2, Cux1, Ctip2, Tbr1).

**Results:** $\Delta O_2$ did not affect brain weight at P7 but reduced cortical thickness in both NR ($p<0.05$) and GR groups ($p<0.001$). $\Delta O_2$ resulted in superficial cortical thinning in both groups and in the deep layers of GR pups ($p<0.001$). Cell density was preserved. $\Delta O_2$ did not affect proportions of callosal, corticothalamic and corticospinal neurons but resulted in a reduction of neurons expressing Cux1 ($p<0.01$) implicated in dendritic branching and synapse formation.

**Conclusion:** Postnatal $\Delta O_2$, a modifiable factor in neonatal care, impairs cortical development in a rodent model with preferential disadvantage to superficial neurons.

**(200 words)**



**Keywords**

Hyperoxia, cerebral cortex, fetal growth retardation, cortical lamination, CUX1 protein.

## 1. Introduction

Advances in obstetric and neonatal care in the last decade have significantly increased survival of premature infants; however, outcome studies describe a high prevalence of neurodevelopmental impairment (Volpe, 2009). Approximately 5-15% of survivors will develop cerebral palsy and around half will have significant deficits in behavioural and cognitive domains (Moster et al., 2008), both of which have considerable educational and economic impact (Saigal and Doyle, 2008). Although the developmental pathways are not fully defined, it is evident that the immature brain is vulnerable to various adverse intrauterine and postnatal exposures during a critical period of growth and maturation and that these adverse exposures may perturb the normal trajectory of cerebral development (Ment et al., 2009).

The layers of the human cerebral cortex start to form from around 7 weeks gestation with newly generated neurons migrating from the ventricular surface to take up position in the cortical layers in a deep to superficial temporal sequence (Sidman and Rakic, 1973)(Figure 1); layer VI is established first followed by layers V, IV, III and II. This "inside-out" pattern of development occurs in both rodents and humans (Rakic, 2007). The deep layers (V and VI) contain large projection neurons which project to subcortical targets, and the superficial layers (IV-II) contain smaller neurons that form more localised intra-cortical connections (Bystron et al., 2008; Molnar and Cheung, 2006). Even after neurons reach their cortical target, dendritic maturation and synaptogenesis continue within the cortical layers throughout gestation. These cells and their projections may, therefore, be vulnerable to environmental effects during the last trimester of gestation, or in the case of preterm birth, during the first weeks of postnatal life within the neonatal unit.

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Fetal growth restriction secondary to placental failure is significant in the aetiology of preterm birth. Preterm growth restricted infants are at increased risk of acute and chronic respiratory morbidities, poor postnatal growth and poor neurodevelopmental outcome in childhood compared to normally grown infants of similar maturity (Yanney and Marlow, 2004). Such infants show a region specific reduction in brain volume on imaging, with a predilection for cortical grey matter (Huppi, 2011).

Respiratory support in the form of supplemental oxygen or mechanical ventilation is inevitably required by preterm infants with acute respiratory morbidity. These therapies are associated with bronchopulmonary dysplasia (BPD), a condition characterised by ventilation/perfusion mismatch, variable oxygen saturations and fluctuations in inspired oxygen. The presence of BPD is an independent predictor of adverse neurodevelopmental outcome. Previous imaging studies of preterm infant brains indicate that respiratory support and supplemental oxygen are risk factors for impaired markers of connectivity and cortical growth which correlate with adverse outcomes in childhood (Boardman et al., 2007). In the experimental setting, supplemental oxygen increases short and long term indices of brain injury (Felderhoff-Mueser et al., 2004; Loeliger et al., 2006; Ratner et al., 2007) while mechanical ventilatory support is associated with poor brain growth, white matter injury and a reduction in neuronal numbers (Loeliger et al., 2006; Verney et al., 2010).

We hypothesised that common perinatal exposures in preterm birth, suboptimal nutrition and fluctuating oxygenation, occurring during a critical period of neocortical maturation, will impair the growth and development of the cortical laminae thus

defining a mechanism for effects on cognitive function. Although previous experimental models designed to examine the relationship between oxygen and the preterm brain have provided important insights into the adverse effects of oxygen, they have traditionally employed high concentrations of oxygen (Ratner et al., 2007; Sirinyan et al., 2006). Such extreme hyperoxaemia is seldom experienced by neonates in contemporary clinical settings where oxygen saturations are targeted to a normoxic range. Despite such targets, preterm infants with ventilation perfusion mismatch and an immature respiratory drive recurrently deviate outside the target range into mildly hypoxic and hyperoxic saturations. The neuropathological consequence of mild deviations is unclear.

Our study aimed to replicate these mild repetitive fluctuations in a rodent model using an oxygen profile which was adapted from transcutaneous arterial oxygen partial pressure ($PaO_2$) measurements of a preterm infant who developed severe retinopathy of prematurity (ROP) and which has previously been validated in animal studies of ROP (McColm and Cunningham, 2000). Our study examines the effects of inspired fluctuating mild hyperoxia ($\Delta O_2$) applied postnatally, and impaired nutrition (initiated during gestation and ongoing after birth), on cortical growth and on the cellular composition of the deep and superficial cortical laminae in the developing brain.

## 2. Material and methods

Animals were studied in accordance with UK Home Office legislation (Animal Scientific Procedure Act 1986; PPL60/12624), the Code of Practice for the Housing

and Care of Animals used in Scientific Procedures, HMSO, 1989, and following Local Ethics Committee approval.

## 2.1 Induction of Growth Restriction

Sprague-Dawley rat dams were fed unrestricted isocaloric synthetic diets of identical colour and texture containing 18% or 9% casein protein from embryonic day 15 (E15) until postnatal day 7 (P7) to induce asymmetrical growth restriction (Bennis-Taleb et al., 1999).

## 2.2 Oxygen profile

The profile was adapted from transcutaneous arterial oxygen partial pressure ($PaO_2$) measurements of a preterm infant who developed severe retinopathy of prematurity (ROP), (McColm and Cunningham, 2000). Continuous recordings of transcutaneous oxygen were taken from the infant over 7 days and averaged every minute. A rat has a circulating $PaO_2$ of 12.9kPa in room air (Penn et al., 1995). It is recommended that preterm infants requiring oxygen have their $PaO_2$ maintained within a range of 6 - 10kPa, mean of 8kPa, i.e. 4.9kPa lower than rat pups (McColm and Cunningham, 2000). Therefore, 4.9kPa was added to each value obtained from the infant profile. The transcutaneous profile was translated into a profile of mildly hyperoxic fluctuating fractional inspired oxygen concentration ($FiO_2$) (Figure 2) around a human equivalent mean of 10kPa. In rats $PaO_2$ varies linearly with FiO2 with little inter-individual variation over oxygen concentrations less than 50%. The computer-controlled system by which the oxygen profile was delivered has been described previously (McColm and Cunningham, 2000).

At birth, litters were randomly allocated to an air or fluctuating hyperoxia ($\Delta O_2$) chamber. The dimensions of both chambers were width 25cm, length 41cm, height 20.5cm. Soda lime was placed in the oxygen chamber to maintain the carbon dioxide at normal atmospheric levels (Medisorb GE Healthcare, UK).

## 2.3 Experimental groups

There were four groups:

1. Control (non restricted diet (NR), reared in room air (RA))
2. Exposure to $\Delta O_2$ alone, NR diet
3. Exposure to growth restricted diet (GR) alone
4. Exposure to both GR & $\Delta O_2$

Temperature was maintained at 21ºC; water available *ad libitum* and light cycled 12 hr on / off schedule. After birth, litters were limited to 10 pups (equal numbers of males & females where possible), weighed; tattooed and placed with mother in RA or $\Delta O_2$ chamber.

## 2.4 Tissue Preparation

At postnatal day 7 (P7), pups were deeply anaesthetized and perfused transcardially with 4% paraformalydehyde. Brains were removed, placed in 4% paraformaldehyde for 24hours, washed in phosphate buffered saline (PBS), cryoprotected in sucrose and sectioned serially in the coronal plane at 20 μm thickness using a Leica CM 1900 Cryostat.

Cryosections rostral to the corpus callosum were matched for each group and stained with cresyl violet. Three sections per brain of the motor cortex and corpus callosum

were imaged on a bright field microscope (Olympus BX51, with a Micropublisher 5.0RTV CCD, Q Imaging) using a x4/0.1 objective.

## 2.5 Immunofluorescence

Sections at the corpus callosum decussation were matched for each group. Primary antibodies were: goat polyclonal antibody to Cux-1 (Santa Cruz Biotechnology, 1:50 concentration), mouse monoclonal antibody to anti-Satb2 (Abcam, 1:25), rat monoclonal antibody to Ctip2 (Abcam, 1:250), rabbit polyclonal antibody to Tbr1 (Abcam, 1:100). Secondary antibodies were: donkey anti-goat (Alexa Fluor-568, 1:200 for Cux1), donkey anti-mouse (Alexa Fluor-488, 1:200 for Satb2), goat anti-rat (Alexa Fluor-568, 1:200 for Ctip2), goat anti-rabbit (Alexa Fluor-488, 1:200 for Tbr1). Tissue sections were dual stained for both Cux 1 / Satb2 and for Ctip2 / Tbr1. TO-PRO-3 nuclear counterstain (Molecular Probes, Invitrogen) was used, Figure 4.

Sections were imaged using a Leica TCS NT confocal microscope (Leica Microsystems, Germany). For Ctip2 / Tbr1 deep layer nuclei, images were acquired using an x10/0.3 Plan Fluortar objective. Serial Z sections were acquired with 4μm between each optical slice through the tissue depth. For Cux1 and Satb2, images were acquired at x10 magnification for deep layers and x20 magnification using a x20/0.7 HC Plan Apo objective for superficial cortical layers with 1.2μm between optical slices in Z. The x20 objective was used for the superifical layers to provide greater lateral and axial resolution required to accurately count the nuclei, due to tge increase in cell density observed in some of the treatment groups in the superificial layers. The higher numerical aperture of the x20 lens (0.7 compared to 0.3) meant that the z distance between optical layers could be decreased from 4 μm to 1.2 μm. Maximum

intensity projections were made from each Z stack using the Leica Lite image analysis software.

### 2.6 Measurements & Assessment of cerebral cortex and corpus callosum

Cortical thickness was measured using Image J software from the outer aspect of layer 1 to the innermost cells of subplate (Figure 3). The thickness of motor cortex and corpus callosum were measured in 3 images per animal and mean values calculated for each animal. Sections were blinded to a single observer (EF) and a random sample checked by a 2nd observer (JW). Inter-observer consistency rate >95% was accepted. Densities of nuclei labelled fluorescently with TO-PRO-3 or with specific antibodies were counted within a 100μm-wide counting frame strip placed in the centre of the motor cortex across the full cortical depth (Figure 4). A minimum of 3 sections from each brain were counted and averaged for. A single observer (EF) was blinded to the study group; accuracy of counts was confirmed on blinded randomly selected recounts by a 2nd observer (PG). Inter-observer consistency was at least 95%.

### 2.7 Statistical Analysis

Continuous variables are reported as mean ± standard deviation (SD) and were analysed for normality by D'Agostino and Pearson omnibus normality test. Statistical analyses were performed using Student's t-test or 2-way analysis of variance (ANOVA) with Bonferroni post-test for multiple comparisons. Non-parametric data is presented as median ± interquartile range. Calculations were made using Prism software, version 5.02. Statistical significance is assumed at p values $<0.05$.

## 3. Results

### 3.1 Oxygen profile (Figure 2)

The mean oxygen concentration delivered over 7 days was 24.9% and ranged from 12.6% to 44.9%. The pups spent 85% of the study period receiving greater than 21% inspired oxygen and 11% of the study period receiving less than 21%.

### 3.2 Body weight

The mean birthweight of NR pups was 6.00g (SD ± 0.68) compared to that of GR pups, 5.26g (SD ± 0.64), $p < 0.001$. At P7, comparing diet alone, NR pups had a mean weight of 16.58g (SD ±1.16) and GR pups had a mean weight of 11.21g (SD ± 0.82), $p<0.001$, demonstrating ongoing postnatal growth restriction (Figure 5A). At P7, exposure to $\Delta O_2$ in the GR group further decreased body weight to 9.98g (SD ± 1.20), $p<0.001$.

### 3.3 Brain weight

At P7, brains of GR pups weighed 0.58g (SD ± 0.04) and were smaller than brains of NR pups (0.64g ±0.03, $p<0.001$)(Figure 6), but demonstrated relative brain sparing in relation to body weight. There was no independent effect of oxygen on brain weight in either group (Figure 5B).

### 3.4 Motor cortical thickness

Despite a decrease in brain weight, total cortical thickness was preserved in GR pups compared to controls (Table 1, Figure 6A, Figure 7). A significant effect of $\Delta O_2$ on total cortical thickness was observed in the $\Delta O_2$ group compared to controls ($p<0.05$). The dual exposure group demonstrated particularly significant cortical thinning in

comparison to both the $\Delta O_2$ group, $p<0.01$, and the GR group, $p<0.001$ (Table 1, Figure 5C, Figure 7).

When thickness of the early born deep cortical layers (V & VI) was considered alone, there was relative conservation of deep layer thickness in the single exposure $\Delta O_2$ and GR groups. Thinning of the deep cortical layers was observed in the dual exposure group, in comparison to the controls, $p<0.001$ (Table 1, Figure 5D, Figure 7).

$\Delta O_2$ adversely affected thickness of the late born superficial layers (IV-II) in both normally grown ($p<0.01$), and growth restricted groups, ($p<0.001$) (Table 1, Figure 5E).

### 3.5 Cell density

Cell nuclei density per square micron ($\mu m^2$) (total TO-PRO-3 stained nuclei $x10^{-3}$ per square micron of cortex) was compared between groups (Figures 8). There was an increase in superficial cell nuclei density observed in the dual exposure group ($p<0.05$) suggesting a reduction in the neuropil in these animals.

### 3.6 Cortical Neurons

#### 3.6.1 .1 Deep layer neurons: Transcription factors Tbr1 and Ctip2

To identify cells in the deep layers of the motor cortex, the transcription factors Tbr1, expressed by corticothalamic projection neurons in layers V & VI, and Ctip2, expressed by corticospinal projection neurons in layer V &VI, were immunofluorescently labelled. The proportion of neurons positively labelled with each subtype transcription factor relative to the total nuclear number stained with TO-

PRO-3, was compared between groups. The proportion of layer V & VI labelled neurons was not affected by exposure to GR or ΔO2 (Figure 10, Table 2).

### 3.6.2 .2 Callosal neurons: Transcription factor Satb2, and callosal thickness

The transcription factor Satb2 is expressed by callosal neurons in the deep and superficial layers. No difference was identified between study groups in the proportion of neurons expressing Satb2 in the deep cortical layers or in the superficial cortical layers (Figure 10, Table 3). In addition no significant effects of GR or $\Delta O_2$ on the thickness of the corpus callosum were observed across groups at a matched point immediately caudal to the decussation of the corpus callosum.

### 3.6.3 .3 Superficial neurons: Transcription factor Cux1

The transcription factor Cux1, expressed by projection neurons in the superficial layers, was studied. The proportion of neurons positively labelled for Cux1, relative to total cells labelled with TO-PRO-3, was compared between groups. $\Delta O_2$ resulted in a reduction in the proportion of Cux1 expressing superficial projection neurons ($p < 0.01$) which was most evident in the context of growth restriction ($p < 0.001$, Figures 9 & 10, Table 4). There was no evidence of Cux1 expression in the layers deep to the superficial cortex.

## 4 Discussion

We have shown, using a clinically derived oxygen profile in a rodent model of growth restriction, that mildly hyperoxic fluctuations adversely affect cortical growth, particularly of the late born superficial cortical laminae, resulting in a thinned cortex,

an overall reduction in cortical cell density within a standard counting frame, and a reduction in the proportion of Cux1 positive neurons.

Cohort data from epidemiological studies suggest that 20-25% of extremely preterm infants develop significant neurodevelopmental impairments in childhood (Saigal and Doyle, 2008). Cerebral imaging studies of preterm survivors show that reduced intelligence and executive function scores correlate with reduced cortical volumes (Soria-Pastor et al., 2009) and with cortical thinning (Skranes et al., 2012) and it is becoming apparent that these specific deficits may be linked to damage in cortical proliferating zones and disruption to the developing neural pathways that are key to higher cognitive function (Volpe, 2009; Ball et al., 2012).

Although the link between cerebral volume and neurodevelopmental sequelae is well established in preterm infants, the mechanisms leading to these deficits are not well defined. Supplemental oxygen therapy is increasingly recognised as a key player in a range of adverse neonatal morbidities, such as retinopathy, necrotising enterocolitis and bronchopulmonary dysplasia, and is an independent predictor of poor brain growth and later neurodevelopmental outcome, including an increased risk of cerebral palsy, reduced IQ and attention deficit disorder (Askie et al., 2003). Nevertheless, a recent multicentre study, which reported a survival advantage of oxygen saturations within a higher targeted range, has influenced clinical practice, despite a concurrent increase in retinal vasculopathy and as yet unreported neurodevelopmental outcomes (Stenson et al., 2011).

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This clinically derived fluctuating oxygen model has become established as a rodent model of retinopathy of prematurity and, although the effect of oxygen flux on the developing brain is recently emerging, in specialised neural tissue such as the retina, such fluctuations have been recognised to be more injurious than constant hyperoxia alone for decades (McColm and Cunningham, 2000; Penn et al., 1995).

In preterm models, a fluctuating profile to 80% inspired oxygen increases indices of brain injury with induction of free radical species and cell death (Felderhoff-Mueser et al., 2004; Loeliger et al., Kuster et al., 2011), but even a more physiological profile of hyperoxia has been shown to result in dysmyelination and an increase in apoptosis with most persistent effects in the cortex (Sedowofia et al., 2008). Moreover recent data from a double hit model of antenatal hypoxia and postnatal hyperoxia show a disproportionate reduction in brain size which is similar to that seen in animals subjected to hyperoxia alone (Gortner et al., 2013). However intermittent *hypo*xia has also been shown to result in permanent hypomyelination with long term sensorimotor deficit (Juliano et al., 2015) and it is increasingly recognised that both hypoxia and hyperoxia may be proinflammatory within the brain (Martin et al., 2011). In our model, animals received additional oxygen (hyperoxia) for 85% of the first week and 11% of the time were in mildly hypoxic conditions. Although our model more closely represents the clinical paradigm than extreme conditions in other studies, the effect of a lesser degree of hypoxia or indeed the rapid swings from hypoxia to hyperoxia may be as important in our data as hyperoxic fluctuations alone.

We found that a mildly fluctuating oxygen profile resulted in a reduction in the thickness of superficial laminae which was accentuated in the context of growth

restriction. The superficial cortical layers in humans have a larger proportion of late-born neurons than other species (Hill and Walsh, 2005) and this allows a more complex architecture of inter-neuronal connections to support vast neuronal communication. This is key to human cognitive ability, a domain that is adversely affected by preterm birth (Soria-Pastor et al., 2009). Cortical thinning was more profound in the late born superficial layers, although deep layer thinning was seen in the group exposed to both growth restriction and hyperoxia. Both these findings are compatible with imaging studies of preterm survivors reporting a reduction in cortical grey matter volumes both in those requiring supplemental oxygen and respiratory support (Boardman et al., 2007; Loeliger et al., 2006; Verney et al., 2010; Anjari et al., 2009), and in those who have suffered intrauterine growth restriction (Huppi, 2011). Given the larger proportion of superficial neurons in humans, it is possible that effects on cortical thickness in rodents are significantly amplified in humans. With birth, the developmental processes of the fetus which normally occur in low oxygen tension, require to undergo immediate adaptation to a relatively hyperoxic environment and which may include additional oxygen. It has been suggested that the normal trajectory of neurodevelopment in such infants may be perturbed by both oxidative stress compounded by a limited antioxidant capacity (Toy et al., 2009) and by modified expression of oxygen-regulated genes (Wellman et al., 2014).

The formation of the cortical laminae is dependent on the precise proliferation and migration of specific cortical neuron subtypes, a process dependent on expression of specific transcription factors which dictate key cellular events specific to the subtype function. The development of cortico-cortical connectivity in humans is most active in the last trimester and continues postnatally, involving completion of migration,

dendrite maturation and synaptogenesis. Cux1 is a recently identified player in cortical development (Cubelos et al., 2010; Li et al., 2010) and expression of this transcription factor specifies superficial neuronal cell identity and directs maturational changes including regulation of dendritic branching and synapse formation with other neurons (Cubelos et al., 2010). Cux1 is therefore crucial to promoting the integration of neuronal circuits in the superficial layers. Genetic knock-out studies of Cux1 and Cux2 in rodents demonstrate reduced complexity of dendritic branching, simpler dendritic tree morphology and reduced dendritic spines, all elements required for inter-neuronal communication and the establishment of cortical circuitry (Cubelos et al., 2010; Cubelos et al., 2015).

In this study, we observed a reduction in the proportion of Cux1 positive neurons in the context of preserved cell density in the superficial layers of animals exposed to fluctuating oxygen. The mechanism underpinning this reduction was not examined in this paper but hypotheses include a functional impairment of Cux1 expression by viable superficial neurons, or a loss of Cux1 expressing cells secondary to neuronal cell death. Absence of Cux1 staining in deeper layers excluded arrested migration of Cux1 positive neurons as a cause of reduced expression. We acknowledge that this study did not examine changes in cortical surface area and thus we cannot comment on effects on total cell number.

Cux1 expression was most significantly affected in the context of growth restriction where cell nuclear density was observed to increase. Within the study model, the underlying mechanism for poor growth includes substrate restriction, inhibition of skeletal growth by maternal glucocorticoids (Holemans et al., 2003) and impairment

of uteroplacental blood flow (Gao et al., 2012) whilst postnatally a reduction in maternal lactation restricts caloric intake (Holemans et al., 2003). Both a reduction in brain weight continuing into adulthood and a reduction in cerebral cortical vascularisation have been previously described (Bennis-Taleb et al., 1999), a phenomenon which may be potentiated by imbalances in angiogenic factors induced by fluctuating hyperoxia. Moreover a reduction in neurotrophic factors in the context of growth restriction, particularly IGF1 (Dhaliwal et al., 2011), and impaired antioxidant capacity may both contribute to a reduction in neuronal cell survival in the context of oxidative injury. There are no other published reports documenting the effect of nutrient restriction on gene expression of cortical transcription factors although an association between protein restriction and increased apoptosis, decreased synaptic density and fewer neurons has been reported in fetal rodents (Liu et al., 2011). Of note we found no difference in the expression of another superficial transcription factor, Satb2, nor any differences in selected deep layer markers.

In conclusion, postnatal fluctuating mild hyperoxia impairs cerebral cortical growth, particularly of the superficial layers, and reduces the proportion of neurons expressing the transcription factor Cux1, specific to superficial neurons, particularly in the context of growth restriction. Although future studies are essential to delineate mechanisms and determine the long term impact, these data which are consistent with the emerging radiological evidence, raise concern that common perinatal exposures may impact on both the specific neural systems involved in cortico-cortical connectivity and the development of higher cortical functioning in survivors of preterm birth.

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## Figure Legends

Colour required for Figure 4, Figure 9 please – all others can be black and white if required.

**Figure 1 – Schematic Illustration of Cortical Development in rats and humans**

Cortical layers labelled with subtype-specific transcription factors explored in this study.

**Figure 2 – Oxygen Profile**

7 day oxygen profile translated from a preterm infant to an inspired concentration for rats. The percentage of oxygen administered to the pups is shown on a minute-to-minute basis. 10080 plots are shown accounting for each minute over a 7 day period.

**Figure 3 – Thickness measurements Cresyl Violet stained motor cortex**

Deep layer thickness measurements were made from a-b and superficial layer thickness measurements made from b-c, where 'a' represents the innermost aspect of the subplate adjacent to white matter, 'b' represents the watershed between the densely packed layer IV cells and the larger more dispersed layer V cells and 'c' the outermost aspect of layer I.

**Figure 4 - Immunofluorescent labelling of rat brain sections**

All images are taken from brains in Control group, ie Room air, 18% protein diet.

**Cortical effects of oxygen and growth**

**a - Deep cortical layers V & VI** - images taken at x10 magnification. 100µm-wide box created at x10 magnification and superimposed on centre of motor cortex. Blue staining: TO-PRO-3 nuclear counterstain; Red stain: Ctip2 (corticospinal neurons, predominately layer V); Green stain: Tbr1 (corticothalamic neurons, predominately layer VI).

**b - Superficial cortical layers IV – II** - images taken at x20 magnification. 100µm box at x20 magnification superimposed on centre of the motor cortex.

Blue staining: TO-PRO-3 nuclear counterstain, Red stain: Cux1 (superficial neurons) and Green stain: Satb2 (callosal neurons).

**Higher magnification images to demonstrate staining on a cellular level:**

**Deep Cortical Layers V&VI, three exemplary pictures for each marker:**

c – Tbr1 stain (corticothalmic neurons) in layer VI alone, d – Tbr with TO-PRO-3 overlay, same region of interest, e – TO-PRO-3 alone for identical region of interest.

f – Ctip2 stain (corticospinal neurons) in layer V alone, g - Ctip2 with TO-PRO-3 overlay, same region of interest, h - TO-PRO-3 alone for identical region of interest.

i – Satb2 stain (callosal neurons) in layer V & VI alone, j – Satb2 with TO-PRO-3 overlay, same region of interest, k - TO-PRO-3 alone for identical region of interest.

**Superficial Cortical Layers IV-II, three exemplary pictures for each marker:**

l – Cux1 stain (superficial neurons) in layers IV-II alone, m – Cux 1 with TO-PRO-3 overlay, same region of interest, n - TO-PRO-3 alone for identical region of interest

o - Satb2 stain (callosal neurons) in layers IV-II alone, j – Satb2 with TO-PRO-3 overlay, same region of interest, k - TO-PRO-3 alone for identical region of interest.

**Figure 5**

**Cortical effects of oxygen and growth**

**A - Body Weight at P7**

2-way ANOVA shows significant interaction effect ($p = 0.012$), and significant effect of both $\Delta O2$ ($p=0.0012$) and growth restriction ($p<0.0001$). Bonferroni post-test analysis was used to compare between study groups, significance is demonstrated on the graphs by significance bars *** $p < 0.001$.

Data shows mean ± standard deviation (SD), n=30 per group.

**B - Brain Weight at P7**

2-way ANOVA shows nonsignificant interaction effect ($p = 0.92$), nonsignificant effect of $\Delta O2$ ($p=0.10$) and a significant effect of growth restriction (***$p<0.0001$).

Data shows mean ± standard deviation (SD), n=30 per group.

**C – E: Data shows mean ± standard deviation (SD), n=9 per group.**

2-way ANOVA confirms variation present in all datasets as described below. Bonferroni post-test analysis was used to compare groups, *$p < 0.05$, **$p<0.01$, ***$p<0.001$.

**C** - Total cortical thickness: layers I-VI (microns) against study group on x axis. 2-way ANOVA shows significant interaction effect ($p = 0.016$), and significant effect of both $\Delta O2$ ($p<0.0001$) and growth restriction ($p=0.02$). Post-test analysis shows no effect of growth restriction (GR) alone. $\Delta O2$ caused a reduction in total cortical thickness *$p<0.05$, with greater effect seen in the dual group, ***$p < 0.001$.

**D** – Deep cortical layers (VI & V): 2-way ANOVA shows significant interaction effect ($p = 0.015$), and significant effect of $\Delta O2$ ($p = 0.003$) but not growth restriction ($p = 0.93$). Post-test analysis shows no effect of $\Delta O2$ or GR alone and reduced thickness in dual group in comparison to GR group only, $p<0.001$.

**E** – Superficial cortical layers (IV-II): 2-way ANOVA shows a non-significant interaction effect ($p = 0.43$), but significant effects of both $\Delta O2$ ($p < 0.0001$) and

growth restriction ($p = 0.0004$). Post-test analysis shows no effect of growth restriction alone but superficial cortical thinning was seen in the fluctuating oxygen group, $^{**}p<0.01$ and in the dual group, $^{***}p<0.001$.

**Figure 6 - Brain from normally grown and growth restricted rat pup**

Gross anatomy of brain from normally grown pup (left) is larger than brain from growth restricted pup (right).

**Figure 7 – Cresyl violet images representing cortical thickness changes observed in each group**

Motor cortex of left hemisphere shown for each study group. Markers for measurements placed at high resolution in Adobe photoshop, version 10.

Where 'a' represents the innermost aspect of the subplate adjacent to white matter, 'b' represents the watershed between the densely packed layer IV cells and the larger more dispersed layer V cells and 'c' the outermost aspect of layer I.

**Figure 8 – Cell Density: Number of nuclei ($x10^{-3}$) per square micron**

Number of nuclei $x10^{-3}$ per square micron of superficial cortical layers against study group.

Data shows mean ± standard deviation (SD), n=9 per group.

2-way ANOVA shows a non-significant interaction effect ($p = 0.28$), a non-significant effect of $\Delta O2$ ($p = 0.097$) but a significant main effect of growth restriction ($p = 0.004$). Bonferroni post-test analysis to compare groups shows:

No significant difference between the control group and $\Delta O2$ group ($p>0.05$).

No difference between the control group and GR group ($p>0.05$).

**Cortical effects of oxygen and growth**

Significant increase in cortical nuclei per square micron in the dual group (* $p<0.05$) compared to control group.

**Figure 9**

**A – Cell density in superficial layers in 4 study groups**

All 4 study groups with TO-PRO-3 counterstain to include superficial layers of motor cortex. 100µm box placed in centre of motor cortex. All 4 study groups demonstrated a preserved total nuclear number in 100µm box in superficial layers (I- IV). In dual exposed group a statistically significant increase in cell density was observed despite cortical thinning.

**B – Cux1 immunostain in 4 study groups**

Superficial layers (I-IV) of motor cortex shown for each study group. 100µm box placed in centre of motor cortex. Box height determined with TO-PRO-3 counterstaining. Cux1 count taken as a proportion of total cells (TO-PRO-3 labelled cells) for each group. Statistically significant reduction in proportion of positively labelled Cux1+ cells observed in ΔO2 group and to a greater extent in dual exposed group. Also note corresponding superficial layer thinning in both affected groups.

**Figure 10 - Deep & Superficial cortical layer labelled nuclei counts**

**A-C Deep cortical layers:**

Proportion of positively labelled neurons with subtype transcription factor relative to total TO-PRO-3 labelled nuclei (y axis) against study group (x axis). Data presented as mean ±SD. 2-way ANOVA demonstrates no significant interaction or main effects in dataset, $p > 0.05$.

**A -** No difference between study groups in the proportion of nuclei expressing Tbr1 (layer VI) in the rodent cerebral cortex.

**B -** No difference between study groups in the proportion of nuclei expressing Ctip2 (layer V) in the rodent cerebral cortex.

**Cortical effects of oxygen and growth**

**C** – No difference between study groups in the proportion of nuclei expressing Satb2 (callosal neurons) in the rodent cerebral cortex in the deep cortical layers.

**D&E Superficial cortical layers**

**D -** Proportion of positively labelled nuclei with Satb2 relative to total TO-PRO-3 labelled nuclei (y axis) against study group (x axis). Data presented as mean ±SD. 2-way ANOVA demonstrates no significant interaction or main effects in dataset, $p > 0.05$. No difference between study groups in the proportion of Satb2 positive nuclei in the rodent cerebral cortex in the superficial cortical layers.

**E –** Superficial Projection neurons expressing Cux1

Data shows mean ± standard deviation (SD), n=9 per group. 2-way ANOVA shows significant interaction effect ($p = 0.0004$), and significant effect of both ΔO2 ($p < 0.0001$) and growth restriction ($p < 0.0001$). Bonferroni post-test analysis shows a significant decrease in proportion of Cux1+ expressing cells in the ΔO2 group (**$p<0.01$) and the dual exposed group, (***$p<0.001$).

**Table 1 – Total Cortical Thickness, Deep layer thickness & Superificial layer thickness, all measurements mean microns ± SD**

| Study Group | Total Cortex (VI-I) | Deep layers (V & VI) | Superficial layers (IV-II) |
|---|---|---|---|
| **RA 18% (Control)** | 1606 ± 118.20 | 988.8 ± 107.70 | 476.1 ± 33.61 |
| **ΔO2 18% (ΔO2 only)** | 1484 ± 120.1 | 970.3 ± 88.75 | 401.1 ± 61.60 |
| **RA 9% (GR only)** | 1610 ± 71.22 | 1059 ± 48.78 | 428 ± 53.52 |
| **ΔO2 9% (Dual)** | 1310 ± 72.56 | 895.9 ± 81.31 | 328.5 ± 26.04 |

**Table 2 – Proportions of Ctip2 and Tbr1 in deep cortical layers**

| Study Group | Ctip2 / Layer V nuclei (mean ±SD) | Tbr1 / Layer VI nuclei (mean ±SD) |
|---|---|---|
| **RA 18%** | 0.28 ± 0.04 | 0.50 ± 0.04 |
| **ΔO2 18%** | 0.27 ± 0.03 | 0.49 ± 0.04 |
| **RA 9%** | 0.27 ± 0.04 | 0.53 ± 0.06 |
| **ΔO2 9%** | 0.27 ± 0.03 | 0.50 ± 0.05 |

**Table 3 - Proportions of Satb2 in deep and superficial cortical layers and corpus callosal thickness**

| Study Group | Satb2 / Layer V &VI nuclei (mean ±SD) | Satb2 / Layer IV-II nuclei (mean ±SD) | Corpus callosum thickness (mean microns ±SD) |
|---|---|---|---|
| **RA 18%** | 0.58 ± 0.01 | 0.67 ± 0.01 | 475.20 ± 32.61 |
| **ΔO2 18%** | 0.59 ± 0.02 | 0.66 ± 0.01 | 538.10 ± 91.28 |
| **RA 9%** | 0.58 ± 0.05 | 0.68 ± 0.02 | 515.20 ± 40.66 |
| **ΔO2 9%** | 0.59 ± 0.03 | 0.66 ± 0.03 | 481.70 ± 97.59 |

**Table 4 – Cux1 positive nuclei in layers IV-II**

| Study Group | Number Cux1 nuclei in 100μm box (mean ±SD) | Total number IV-II nuclei in 100μm box (mean ±SD) | Cux1 number / Total nuclei number (mean ±SD) |
|---|---|---|---|
| **RA 18%** | 215.0 ± 32.22 | 417.0 ± 59.8 | 0.52 ± 0.05 |

| | | | |
|---|---|---|---|
| **ΔO2 18%** | 163.8 ± 18.27 | 355.6 ± 38.2 | 0.46 ± 0.04 |
| **RA 9%** | 206.2 ± 30.47 | 421.8 ± 74.08 | 0.49 ± 0.03 |
| **ΔO2 9%** | 129.9 ± 14.42 | 383.6 ± 41.25 | 0.34 ± 0.03 |

Figure 1
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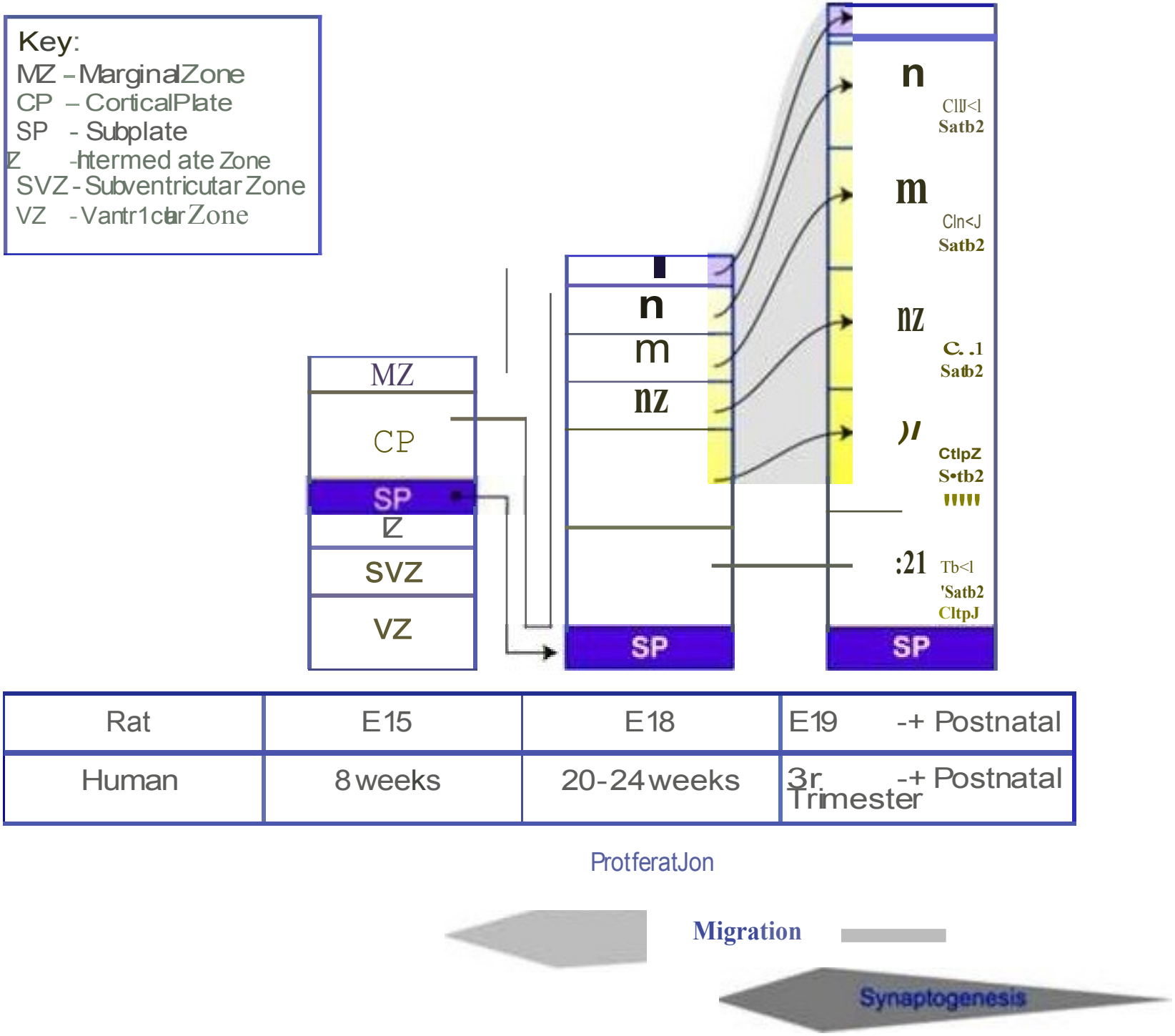


| Rat | E15 | E18 | E19 -+ Postnatal |
|---|---|---|---|
| Human | 8 weeks | 20-24 weeks | 3r Trimester -+ Postnatal |

ProtferatJon

Migration

Synaptogenesis

Figure 2
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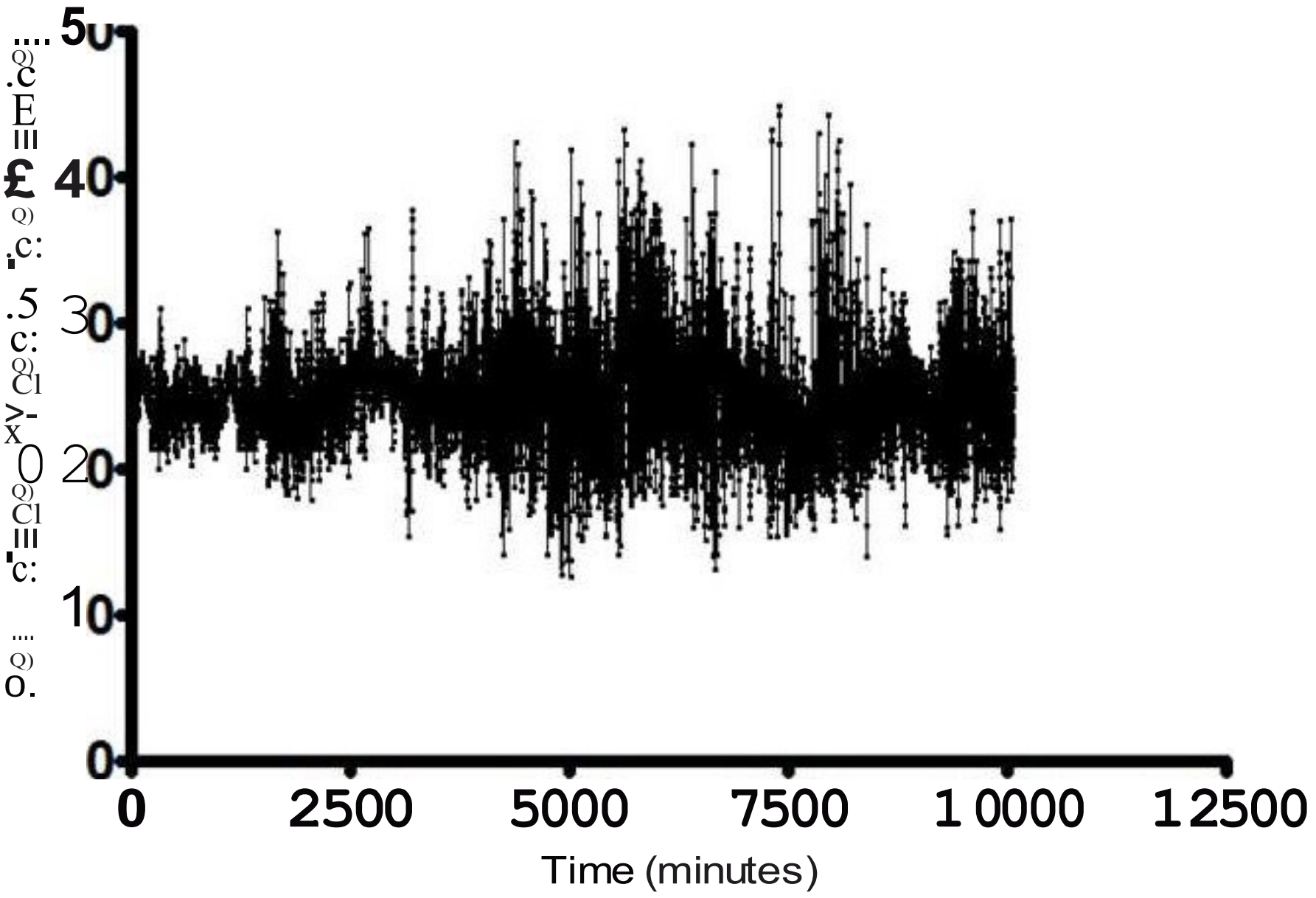

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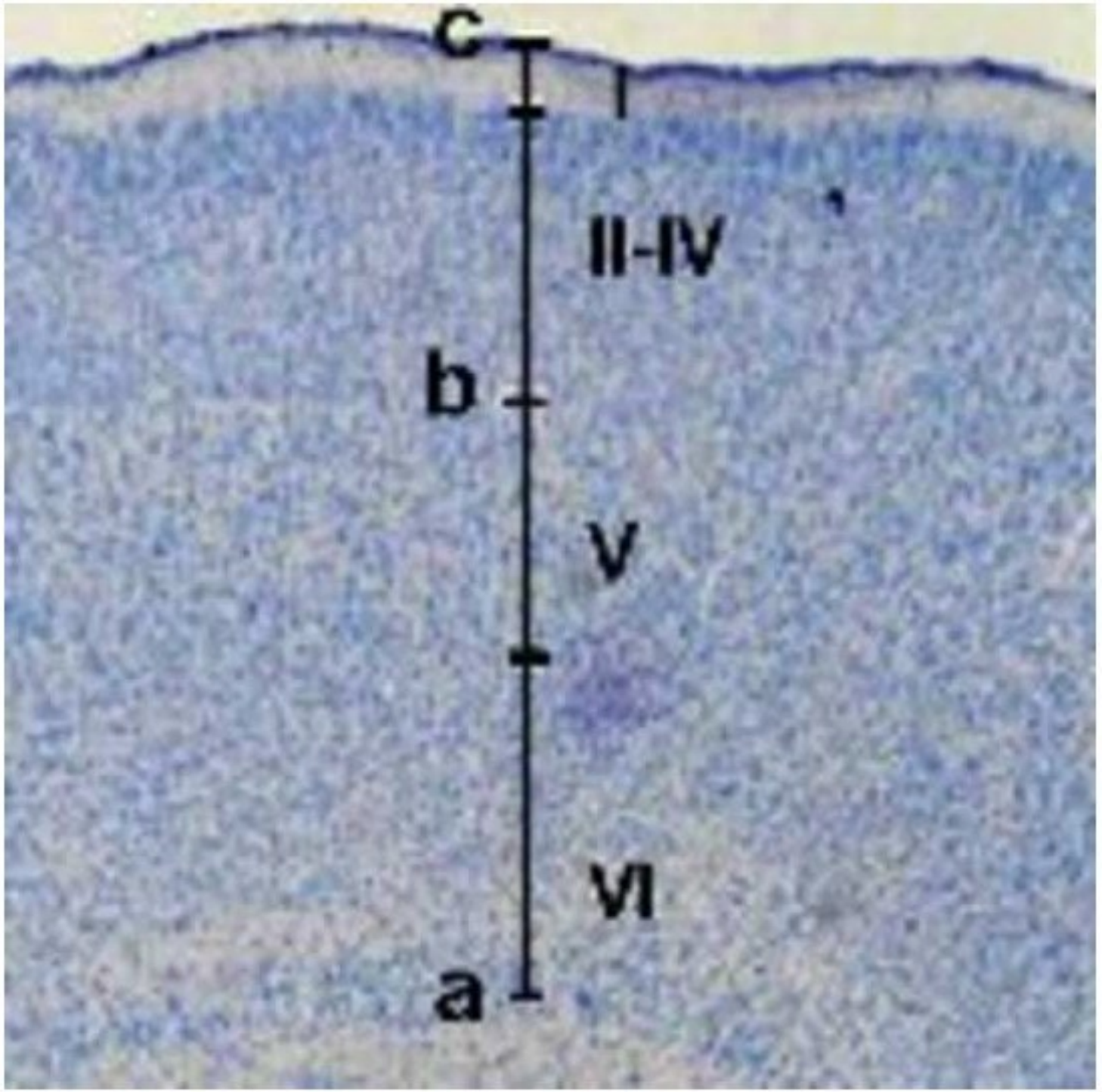

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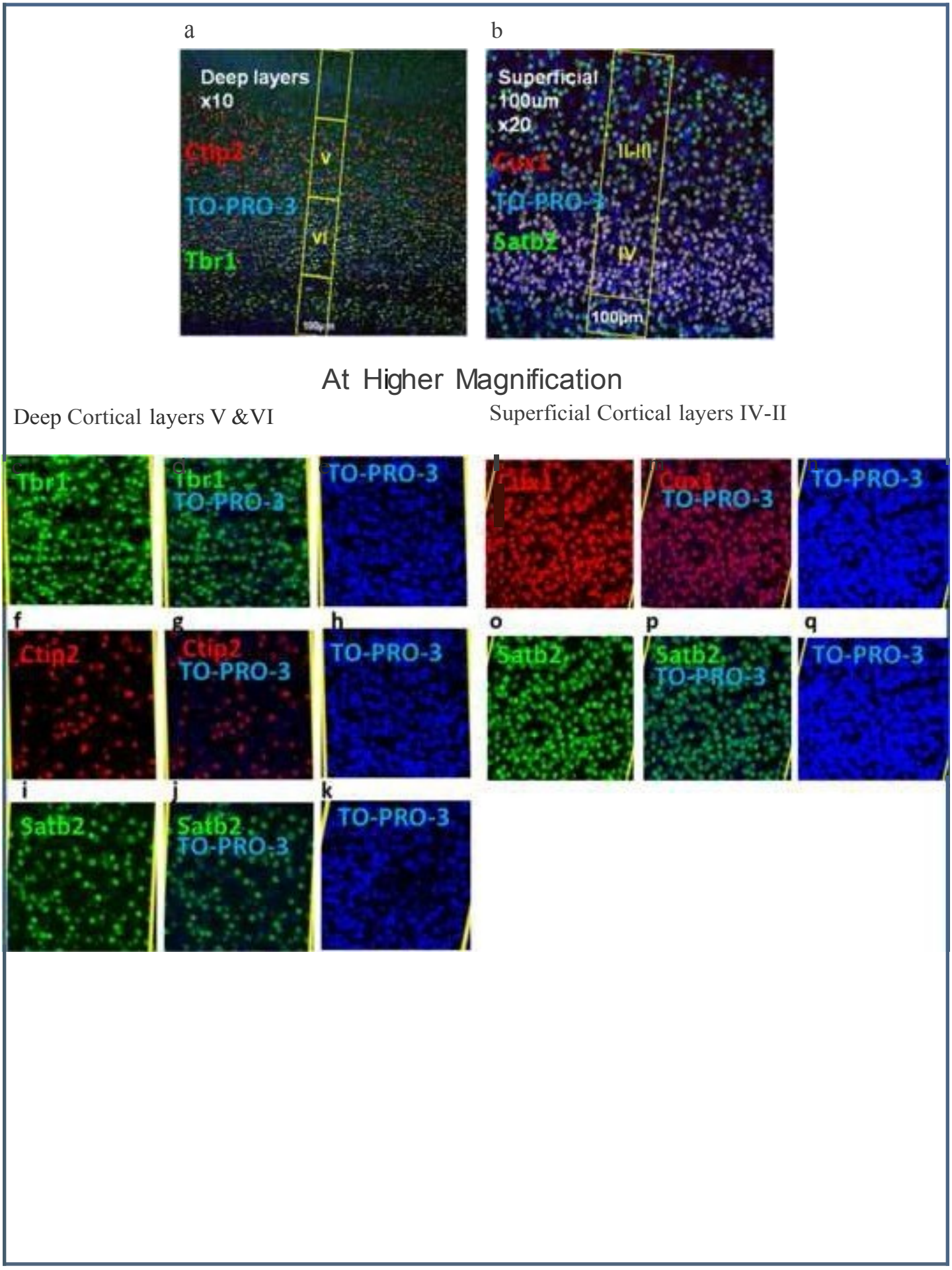

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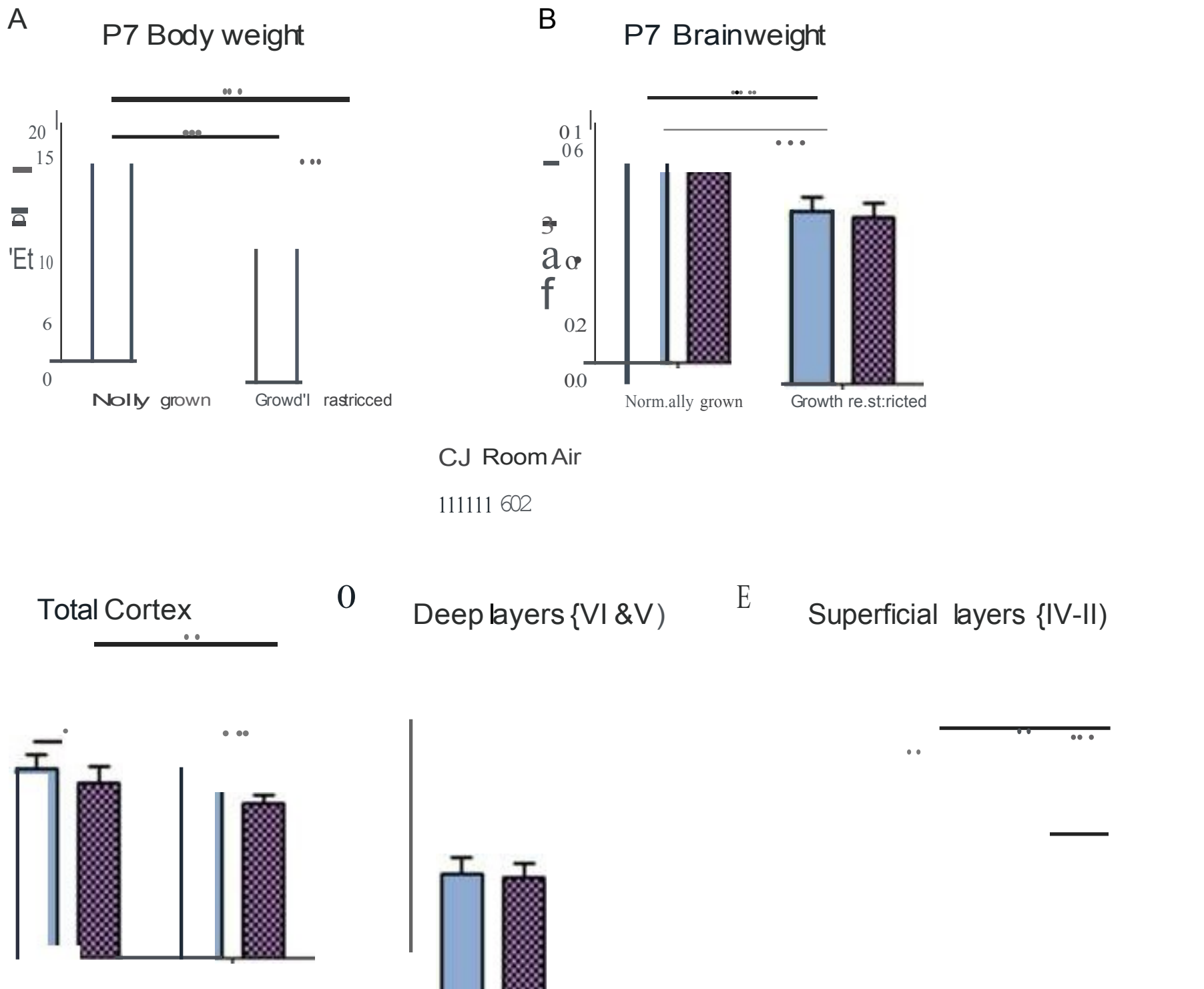

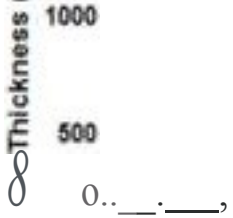


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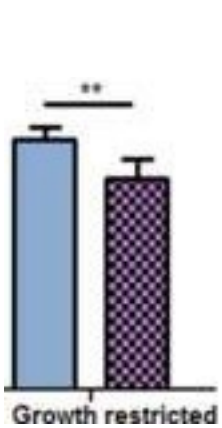


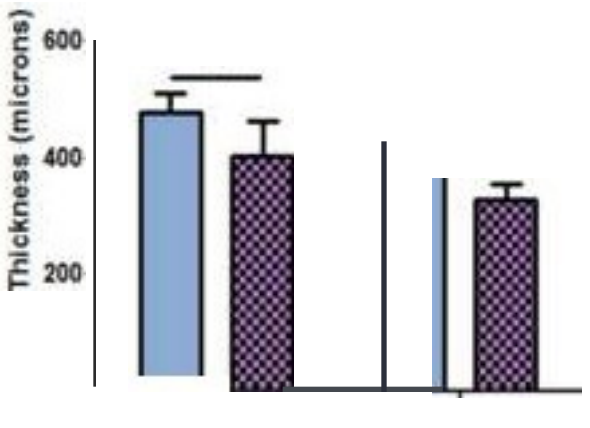

Figures

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Figure 7
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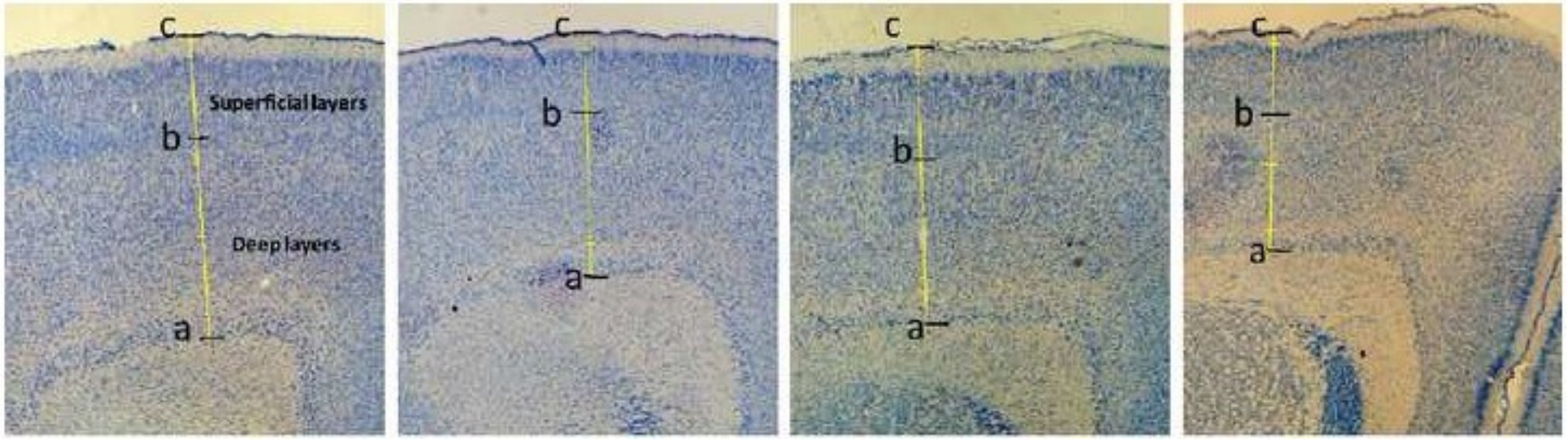


Control

02 Only

- Total cortical thinning
- Superficial thinning

GR only

Dual

- Total cortical thinning

Deep thinning

- Superficial thinning

Figures

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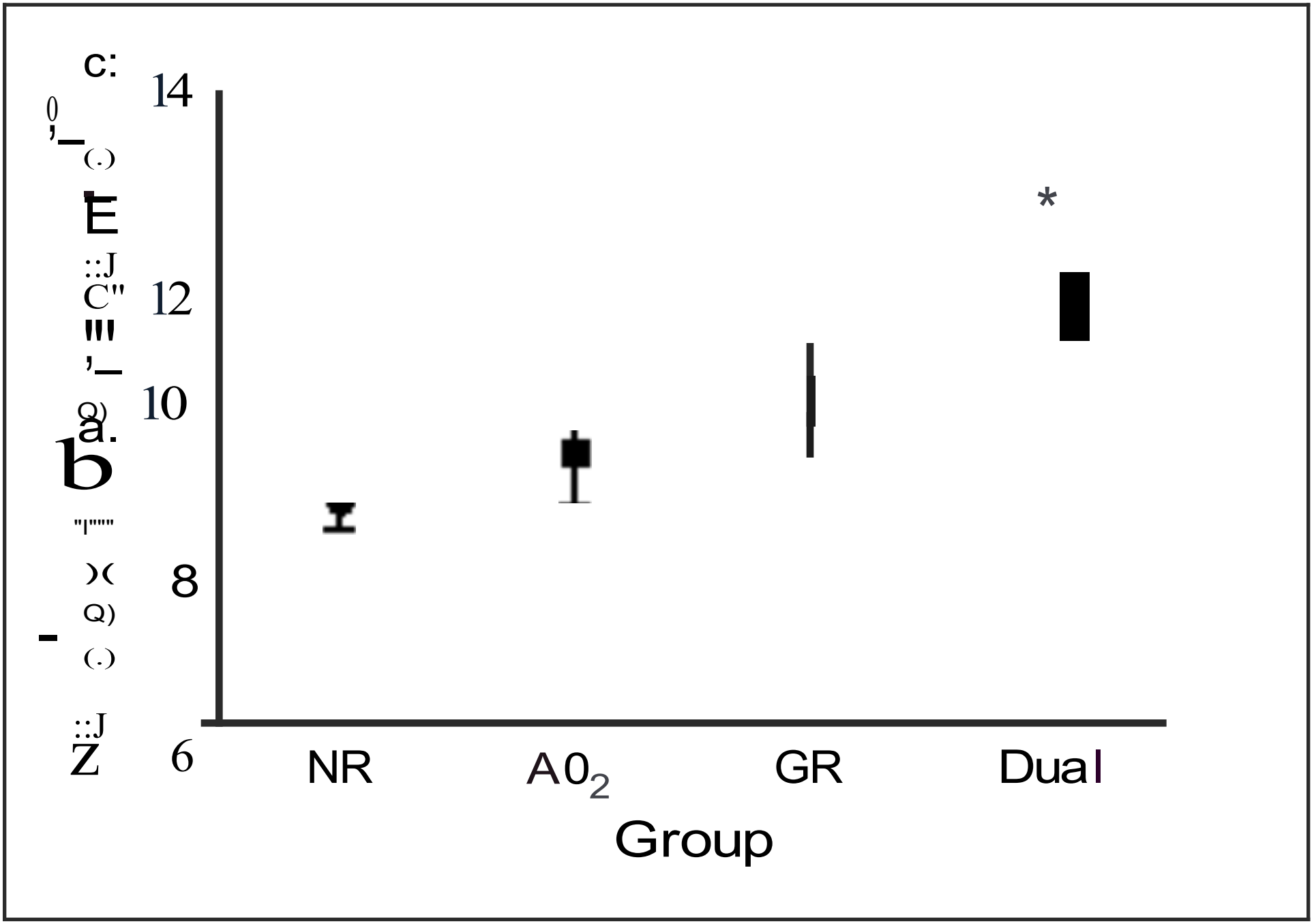

Figures

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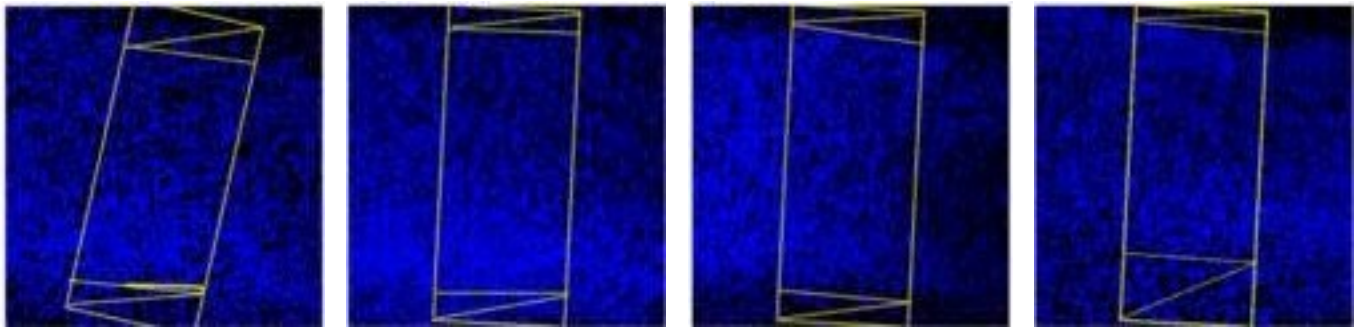
## A. TO-PR0-3 staining Superficial layers



Control | 1102 Only | GR only | Dual

t Celldensity (nudei/ IOOT)

## B. Cuxl staining Superficial layers

Control | 1102 Only | GR only | Dual

I propofl ion Cuxl+ staiing

I .propofl lon CUxl + staiing

Figure 10
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